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Quantification of L-Dopa and Dopamine in Squid Ink: Implications for Chemoreception

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Abstract. Squid ink is an alarm substance that both confuses predators and alerts conspecifics to the presence of danger. Although the ejection of ink is a powerful visual stimulus, studies also indicate a chemical component to the signal. Squid ink is composed mainly of melanin pigments, but the nonpigmented portion of the ink contains the enzymes and precursors of melanin synthesis. Our previous behavioral studies showed that squid olfactory organs detect L-dopa, a key chemical in melanogenesis. Squid olfactory neurons also respond to dopamine, a biogenic amine not previously described in squid ink. We performed HPLC on ink taken from the ink sacs of adult *Loligo opalescens*. The ink was conjugated with orthophthaldialdehyde (OPA) and injected into the HPLC, and amine-containing compounds were detected fluorometrically. Standard curves constructed for L-dopa and dopamine allowed quantitation from individual ink sacs. We found that L-dopa was present in undiluted ink at a mean concentration of 1.15 m*M* and was significantly greater than the mean dopamine concentration of 0.19 m*M*. These values are greater than those at which both compounds are effective in behavioral and electrophysiological experiments. In addition we found that an unidentified antioxidant in the ink may prevent rapid oxidation of L-dopa and dopamine following dilution in seawater.

Introduction

Inking by cephalopods has long been recognized as an adaptive response to predation and physical threat. The ink clouds produced by these animals allow for retreat from a threatening situation and leave behind either a diffuse "smoke screen" or a compacted, long-lasting decoy that serves as an effective visual distraction for predators. Ink may also contain compounds that interfere with a predator's chemosensory abilities, although the behavioral evidence is largely anecdotal (Fox, 1976; MacGinitie and MacGinitie, 1968). In support of these ideas, Prota *et al.* (1981) found a high specific activity of tyrosinase in cephalopod ink and suggested that the *in situ* oxidation, by this enzyme, of phenols in the ink to quinones may be a source of the hypothesized chemical messengers.

In addition to affecting predators, ink clouds may also alert conspecifics to danger, especially within the dense schools of many squid species. Furthermore, the warning signal is likely to be chemical as well as visual because the visual component would be of limited usefulness at night or in the dark ocean depths. In this respect, ink would play a pheromonal role, because its release and detection could provide a basis for intraspecies communication. Indeed, local application of diluted squid ink to the olfactory organ of restrained squid caused strong escape jetting responses (Gilly and Lucero, 1992). In addition, 1-3, 4-dihydroxyphenylalanine (L-dopa), a colorless compound found in cuttlefish ink (Jimbow *et al.*, 1984), elicited escape responses in the absence of other metabolites of melanogenesis including tyrosinase, phenols, or quinones. Therefore, without ruling out effects of the other metabolites, initial behavioral experiments implicated L-dopa as a potential chemical cue (Gilly and Lucero, 1992). L-dopa is an intermediate, not only in the synthesis of melanin and related pigments, but also of dopamine and other neurotransmitters (Waite, 1992). Thus, preliminary physiological studies were performed and showed that diluted squid ink, L-dopa, and dopamine all inhibited spontaneous firing in primary sensory neurons isolated from squid olfactory organs (Lucero *et al.*, 1992).

Because L-dopa and dopamine both mimic the effects of squid ink on primary sensory neurons, we postulate

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that ejected ink functions as a warning signal, and that the two metabolites are effector molecules. Neither substance, however, has ever been identified in squid ink. Therefore, we used high pressure liquid chromatography (HPLC) to identify and quantify L-dopa and dopamine in the ink from individual ink sacs. We report here that squid ink contains free L-dopa and dopamine in concentrations sufficient to produce physiological effects. Moreover, the ink also appears to contain an antioxidant that retards the rapid oxidation of these compounds in seawater.

Materials and Methods

HPLC apparatus and fluorescence detection

Chromatography was performed with two Waters pumps connected to a Waters Model 720 System Controller programmed to generate a dual-solvent, stepped-elution profile. Peaks were detected by a Fluoro-Tec digital filter fluorometer with an excitation wavelength of 320 nm and an emission wavelength of 450 nm. Peak areas were integrated by a Waters Data Module. Separation of ortho-phthaldialdehyde (OPA)-derivatized primary amines was performed on an Ultrasphere 3-μm Octadecasilane (ODS) column of 75 mm length and 4.5 mm internal diameter (Jaeckle and Manahan, 1989; Manahan, 1989).

A combination of aqueous and nonaqueous solvents was used to create an elution profile with a total run time of 23 min. The aqueous solution (solvent A) contained 150 mm sodium acetate (pH 7.2), 9.5% methanol, and 0.5% tetrahydrofuran (THF). The nonaqueous solution (solvent B) was 100% methanol.

A modified stepped-elution profile was developed that allowed for the clear separation and signal integration of L-dopa and dopamine at a flow rate of 1.8 ml/min. The time course for solvent B was as follows: constant 10% from 0 to 1 min, linear increase to 20% from 1 to 4 min, constant 20% from 4 to 9 min, linear increase to 50% from 9 to 13.5 min, constant 50% from 13.5 to 19 min, linear increase to 90% from 19 to 21 min, linear decrease to 10% from 21 to 23 min. At 23 min, the initial conditions were reattained and the column was ready for the next injection. The elution times for L-dopa and dopamine under these conditions were 7.82 $\pm$ 0.08 (SD; n = 19) and 13.25 $\pm$ 0.08 min (SD; n = 18), respectively.

Electrochemical detection

An additional HPLC column and detection method was used to verify results obtained with the OPA method. HPLC separation of diluted squid ink was performed on a C-18 reverse phase column (Rainin) in phosphate-buffered mobile phase consisting of monosodium phosphate ($Na\text{-}H_2PO_4$), 50 m*M;* ethylenediaminetetraacetic acid (EDTA), 200 μM; and heptane sulfonic acid, 1.2 m*M;* pH 3.6. Biogenic amines in the ink were detected electrochemically with an ESA Coulochem (Model 5100A; 5021 conditioning cell; 5011 analytical cell) in the reductive mode (Gomez-Nino *et al.*, 1990).

Standards and standard curves for fluorescence detection

Because of their low solubility and rapid oxidation, 50 μm L-dopa and dopamine standards were made up in a solution of 10 mm ascorbic acid and 150 mm sodium acetate (pH 10). As an added measure to prevent oxidation during preparation, all solutions were bubbled with N_2 gas, placed on ice, and used within 6 h.

All samples were filtered (0.2 μm Millipore) prior to derivatization, which was carried out by adding 25% OPA by volume to the injection sample. The derivatized samples were then vortexed briefly and centrifuged (14,000 $\times$ g) for 1 min. Volumes of supernatant, ranging from 20 μl to 400 μl, were injected for analysis within 3 min of derivatization. An example of HPLC traces obtained for 100-μl injections of 50 μm L-dopa and dopamine is shown in Fig. 3A. Chemicals were obtained from Sigma (St. Louis, MO) and were HPLC grade or ACS reagent grade.

Standard curves (data not shown) were established over the range of injections that produced the best integrated peaks. The L-dopa curve was based on 19 injections with injection volumes corresponding to L-dopa amounts ranging from 0.5 to 5.0 nanomoles. The dopamine curve was based on 18 injections with the lower limit of detection at 1.25 nanomoles. Each curve was estimated by linear regression, and the r^2 values were 0.999 and 0.986 for L-dopa and dopamine, respectively.

Standards for electrochemical detection

The standards used for electrochemical detection were made fresh daily in 1 N perchloric acid (PCA) and included L-dopa, dopamine, dihydroxyphenyl acetic acid (DOPAC), and 3,4-dihydroxybenzylamine (DHBA). DOPAC is a catabolite of dopamine that occurs naturally in squid ink. DHBA was added, as an internal control, at the time of extraction of ink samples being prepared for electrochemical detection (see below). Electrochemical detection of these four standards is exemplified in Figures 2A (50 ng each) and 2B (100 pg each). Standards were run and calibrated at two different gain settings so that the L-dopa and dopamine peaks from ink could each be integrated at optimal sensitivities.

Extraction and preparation of squid ink for fluorescence detection

Live specimens of adult *Loligo opalescens* were collected in Monterey Bay, California. Squid were decapi-

tated, ink sacs were removed by dissection, and whole ink was manually recovered.

An intact ink sac was placed on a sheet of Parafilm, and the ink duct was transected about 5 mm anterior to its emergence from the sac. A small glass rod was then used to gently express the ink onto the Parafilm. Simultaneously, one end of a sterile 10-μl microcapillary tube was introduced into the emerging ink and allowed to fill halfway by capillary action. Several tubes were necessary to collect the ink from a single sac. Nitrogen gas was used to blow the ink out of the tubes and into a preweighed Eppendorf vial that contained 1 ml of 150 mM sodium acetate and 10 mM ascorbic acid. The vial was immediately reweighed and put on ice. This procedure allowed us to rapidly collect and weigh most of the ink within an ink sac and simultaneously to dilute it into a solution containing an antioxidant, thereby minimizing the risk of oxidation. Only ink collections yielding more than 15 mg of ink from a single squid were used in the quantitative analysis of L-dopa and dopamine.

The vial containing diluted ink was centrifuged (14,000 $\times g$) for 10 min at 4°C. The ink supernatant was removed and centrifuged in 0.2 μm filter tubes for 45 s. The filtered ink supernatant was conjugated with OPA in the same manner as described for the dopamine and L-dopa standards above.

Preparation of ink for electrochemical detection

In experiments where the electrochemical detection method was used, the ink sac was blotted dry and placed between a fold of parafilm with the ink duct extending into the collection tube. The parafilm was gently pressed, and drops of ink fell directly into an iced 1.5-ml tube containing 0.5 ml of the acetate/ascorbate buffer and 50 μg/ml DHBA. After 6 samples were collected, the tubes were vortexed for 30 s and immediately frozen on dry ice for shipment from Monterey, California, to Salt Lake City, Utah. Samples were kept frozen at −70°C for 3 days, quickly thawed, vortexed briefly, and run through alumina silica columns (Gomez-Nino *et al.*, 1990). L-dopa and catecholamines in the ink sample were eluted from the alumina column with 1 N PCA and stored at 4°C for 1–4 days. The alumina procedure was necessary to remove ascorbic acid from the ink because, as a reducing agent, ascorbic acid interferes with the oxidation-reduction reaction used in electrochemical detection (Gomez-Nino *et al.*, 1990).

Quantification of L-dopa and dopamine in ink by fluorescence detection

Several different volumes of a given sample were injected into the HPLC so that a range of well-integrated peaks could be obtained. The first injection volume for each ink sample was 100 μl, and the size of the resultant peaks guided the selection of the next injection volume. Whenever possible, replicates were injected at one particular volume to establish a measure of injection error. The standard curves for dopamine and L-dopa were used to transform the integrated peak areas from each analysis of ink into nanomolar quantities that were then corrected for injection volume and dilution to obtain millimolar concentrations that refer back to the original ink sample.

Quantification of L-dopa and dopamine in ink by electrochemical detection

Standards containing known amounts of L-dopa, dopamine, DHBA, and DOPAC were separated on the HPLC, and peaks were integrated and calibrated according to the known amount in the standard. The Waters Data Module was programmed to integrate and quantify peaks from ink samples having the same retention times as the standards. The amounts of biogenic amines in each ink sample were corrected for oxidative loss through the alumina column by a factor of 3.13 relative to the internal standard (DHBA). We were unable, however, to correct for the loss that probably occurred between the time the samples were thawed and run on alumina columns and the time of their injection into the HPLC.

Results

Identification of L-dopa and dopamine in squid ink

Two methods were used to identify the HPLC peaks corresponding to L-dopa and dopamine in the ink. One was to compare the profile and elution times of the peak with those of the standards, and the second was to 'spike' the ink by adding 4 nanomoles of L-dopa and dopamine to the ink sample. The HPLC trace of fresh ink from a single ink sac diluted with the antioxidant ascorbic acid is shown in Figure 1A. Several small unidentified peaks precede and follow the large L-dopa peak. The sizes of these smaller peaks in ink samples from different squid varied, as did the size of the dopamine peak.

Addition of L-dopa and dopamine to the ink is shown in Figure 1B. The areas of the L-dopa and dopamine peaks were dramatically increased without affecting the overall shapes or elution times of any of the other peaks in the sample. Thus, the L-dopa peak in ink had an average elution time of 8.08 ± 0.15 min (SD, n = 47), and the dopamine elution time averaged 13.49 ± 0.26 min (SD, n = 41). These values were very close to the elution times for the L-dopa and dopamine as cited above. Similarly, when preparations of ink and standards were run on the reverse phase C18 column and detected electrochemically, the ink samples had peaks that eluted at the same time as the standards. In Figures 2A and C, the gain of the

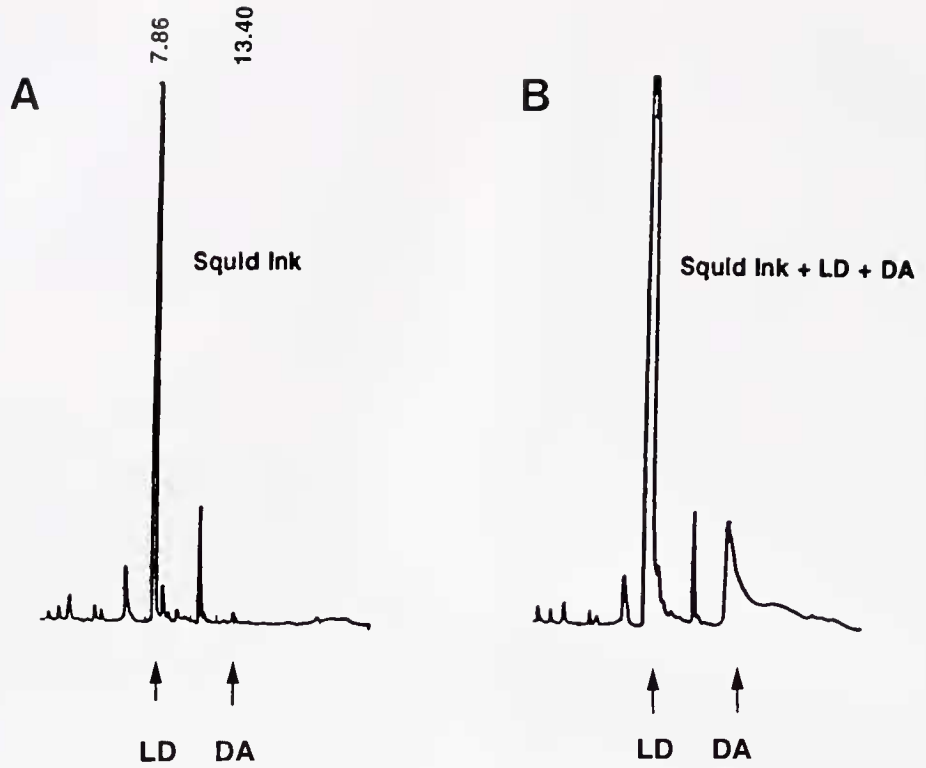

Figure 1. Primary amines were identified in squid ink by 'spiking' with L-dopa (LD) and dopamine (DA). (A) The HPLC trace obtained by injecting 50 μl of diluted squid ink conjugated with OPA (as described in the Methods) shows a prominent L-dopa peak at 7.86 min and a small dopamine peak at 13.4 min. (B) 50 μl of a 'spiking' cocktail containing 250 μl of diluted squid ink, 25 μl of L-dopa, and 25 μl of dopamine was injected into the HPLC to positively identify the L-dopa and dopamine peaks. Both peaks increased in area with little change in elution time.

electrochemical detector was set to 100, and an L-dopa peak can be seen at 4.72 min in both the standard and in the ink. The peaks for dopamine and DOPAC in ink are not resolvable at this gain setting, but a bump at 16–17 min is discernible. With the gain setting turned up to 9900, the dopamine and DOPAC peaks in the same sample of ink are clearly resolved (Fig. 2D), but the L-dopa peak (4.74 min) is now saturating. As mentioned in the Methods, 50 μg/ml DHBA was added to the ink as an internal standard and appears as the large saturating peak in Figures 2C and 2D at 10.37 and 10.34 min respectively.

Several amino acids and neurotransmitters were run as controls, either with fluorescence or electrochemical detection methods, to test whether their elution times differed from those of L-dopa and dopamine. None of the substances tested (aspartate, glutamate, arginine, tyrosine, taurine, phenylalanine, ammonia, ammonium, octopamine, histamine, and norepinephrine) overlapped with the L-dopa or dopamine peaks, but the retention times of some of the amino acids and amines were similar to those of other peaks in the ink sample, suggesting that these substances may also be present in ink.

Quantitative analysis

After correcting for the injection volume and dilution of the ink, the mean concentrations of L-dopa and dopamine were obtained for each ink sac analyzed by the fluorescence method (Table 1). The number of injection volumes for a given ink sac (n) is less for dopamine than L-dopa, because several of the injection volumes used to obtain an L-dopa peak contained less than the minimum detectable amount of dopamine (1.25 nanomoles). Integration of the dopamine peaks in such profiles was deemed unreliable, and the resulting values were not used for further analysis.

The concentration of L-dopa in all of the ink sacs analyzed with the fluorescence detector averaged 1.15 $\pm$ 0.56 mM (n = 10), and the dopamine concentration averaged 0.19 $\pm$ 0.07 mM (n = 9). The highest values for each substance, approximately twice the means, were found in ink sac G. The coefficient of variation (CV), or percent error, in measuring the L-dopa and dopamine in individual ink samples by fluorescence detection averaged 3% (CV = SD/mean $\times$ 100%). This

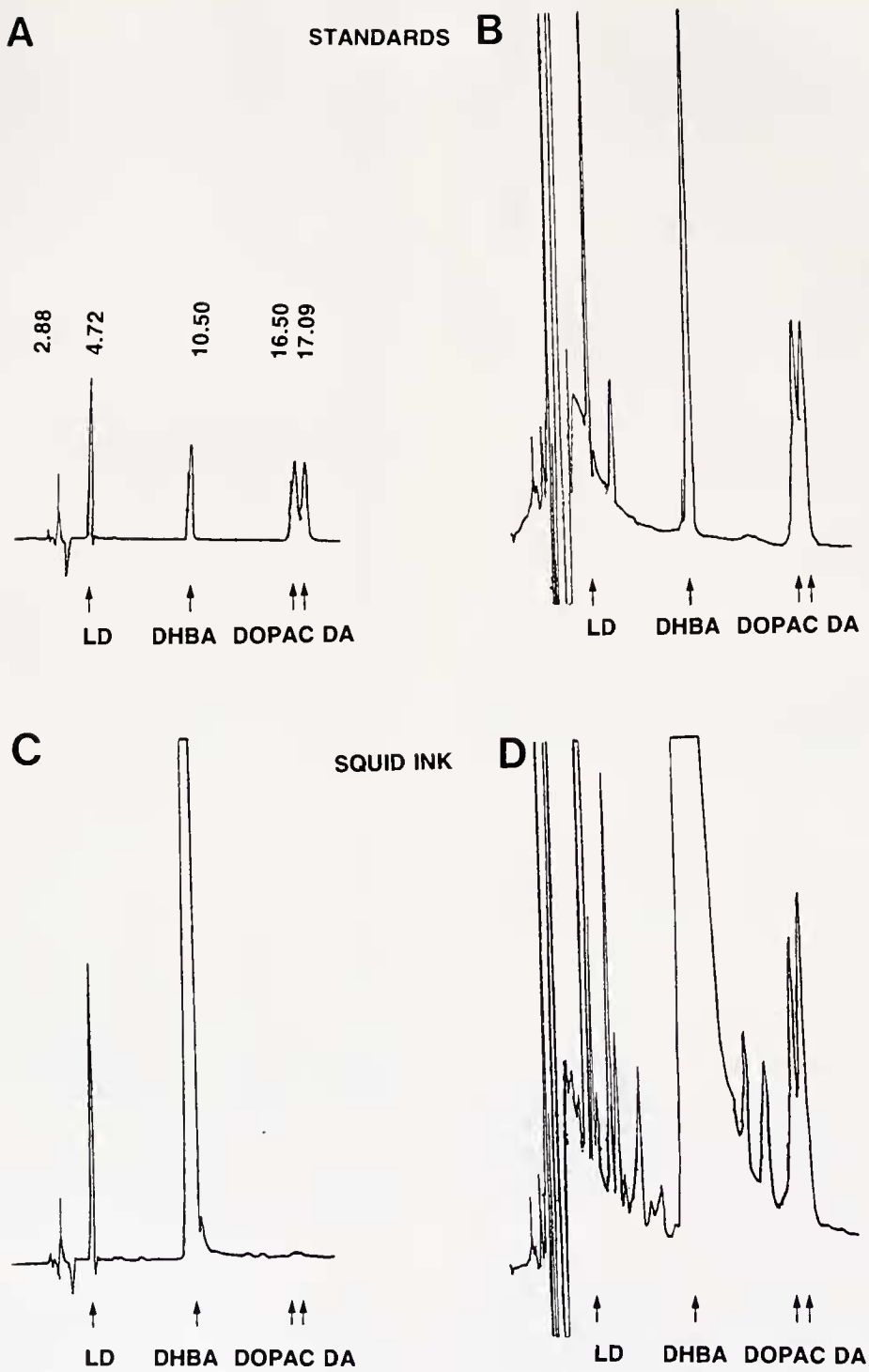

Figure 2. Electrochemical detection verifies the presence of L-dopa (LD) and dopamine (DA) in squid ink. (A) The HPLC trace of a 10 μl injection of standard solution containing 500 pg/μl each of DA, DOPAC (dihydroxyphenyl acetic acid), DHBA (3,4-dihydroxybenzylamine), and LD was obtained at a gain setting of 100. (B) The standard solution was diluted to a concentration of 10 pg/μl and the 10-μl injection is shown at a gain setting of 9900. The initial peaks at about 2 min in all traces represent the solvent front reaching the detector. (C) The HPLC trace of 20 μl of squid ink prepared for electrochemical detection (see Methods) recorded at the same gain as in (A) shows a large L-dopa peak at 4.72 min and a saturating DHBA peak at 10.37 min. The small DOPAC and dopamine peaks were not integrated. (D) The HPLC trace of 10 μl of squid ink from the same sample as in (C) recorded at a gain of 9900, shows that, although multiple peaks are visible at this sensitivity, the L-dopa, dopamine, DHBA, and DOPAC peaks are easily recognized.

Table I

Concentrations of L-dopa and dopamine for ink from individual ink sacks

	L-Dopa					Dopamine				
Ink sac	Mean conc. (m*M*)	(±)	SD	CV (%)	*n*	Mean conc (m*M*)	(±)	SD	CV (%)	*n*
A	0.812		0.034	4.2	7	—		—	—	—
B	0.598		0.035	5.8	5	0.183		—	—	1
C	0.615		0.022	3.6	6	0.081		0.003	3.7	2
E	1.967		0.098	5.0	5	0.249		0.002	0.8	2
F	1.557		0.054	3.5	3	0.160		0.006	3.7	2
G	2.001		0.062	3.1	3	0.268		0.006	2.2	2
H	1.513		0.022	1.4	4	0.249		0.004	1.6	2
I	0.339		0.004	1.2	4	0.083		0.000	0.5	2
J*	0.967		0.015	1.5	2	0.168		—	—	1
K*	1.092		0.097	8.9	2	0.248		0.001	0.4	2

	Mean of Mean Conc. (m*M*)	(±)	SD	CV (%)	*n*
L-Dopa	1.146		0.556	48.5	10
Dopamine	0.188		0.067	35.9	9

Aliquots of diluted ink from 9 ink sacs were analyzed by HPLC and quantified using the appropriate standard curve and correcting for injection volume and dilution; *n* = number of injections at various volumes.

* Ink was diluted and frozen for 3 weeks at −70°C before HPLC analysis.

value was similar to the CV measured for dopamine and L-dopa when constructing each standard curve, suggesting that the measurements of ink samples from a given ink sac are at least as accurate as the measurements made from stock solutions.

Much higher variability existed between ink sacs, the CV being 49% for L-dopa and 36% for dopamine. But this variation does not appear to be random, because the highest values for both L-dopa and dopamine, about twice that of the means, were found in ink sac G; and the lowest values were found in ink sac I. The concentrations of L-dopa and dopamine found in ink sac I (L-dopa, 339 μM; dopamine, 83 μM) are similar to those found in one ink sample by using the electrochemical detection method (318 μM and 25 μM respectively). The average concentrations, measured with the electrochemical detection method, of L-dopa and dopamine in ink were 162.8 ± 90.2 μM (n = 6) and 12.0 ± 7.0 μM (n = 6), respectively. Presumably, these slightly lower concentrations reflect slow oxidation of the ink before analysis (see Methods and below). The CV for measurements within an ink sample was higher than with the fluorescence method at 7%. As with fluorescence detection, there was a large degree of variability between ink samples (CV for L-dopa = 55%; dopamine/DOPAC = 58%).

Oxidation experiments

One possible explanation for the high variability between ink samples is that different amounts of oxidation of L-dopa and dopamine occurred during the preparative steps leading to HPLC analysis. To explore this idea, we studied the stability of biogenic amines, in stock solutions and in ink, that were diluted directly into sterile filtered seawater (0.2 μm Millipore filter), with and without the antioxidant, ascorbic acid.

First, Figure 3A shows the HPLC traces for control injections of 100 μl of 50 μM stock solutions of L-dopa and dopamine plus 10 mM ascorbic acid, at time zero and after 10.5 h at room temperature. There was only a 4% decrease in the amount of L-dopa and an 18% decrease in dopamine, indicating that ascorbic acid prevented oxidation of the stock solutions.

Second, stock solutions of L-dopa and dopamine (50 μM) were made up in sterile filtered seawater without ascorbic acid. At time zero (Fig 3Ba), a 200-μl injection of sample resulted in highly attenuated peaks for both L-dopa (82% reduction) and dopamine (52% reduction), relative to control data in Figure 3A. After 68 min, neither L-dopa nor dopamine were detectable, and both compounds thus appeared to be completely oxidized (Fig. 3Bb).

In contrast to the rapid and complete oxidation of L-dopa and dopamine stock solutions in seawater, dilution of squid ink into sterile filtered seawater decreased L-dopa by 15% and dopamine by only 1% (relative to time zero values), after 93 min at room temperature, including 25 min of gassing with oxygen (Fig. 3C). These results suggest that squid ink contains an antioxidant that protects L-dopa and dopamine from rapid oxidation. Moreover, the large variation in the amounts of L-dopa and dopamine among different ink sacs is not likely to be due to oxidation during ink collection or preparation for fluorescence analysis. In contrast, running samples through the alumina column for electrochemical detection (see Methods) will remove reducing agents from the ink. This difference in ink preparation may account for the lower average concentrations of L-dopa and dopamine found by electrochemical detection.

Discussion

This work represents the first quantitation of catecholamines in ink obtained from individual squid ink sacs and the first identification of dopamine in cephalopod ink. Although L-dopa has previously been identified in cuttlefish ink (Jimbow *et al.*, 1984), the reported units do not allow quantification of the concentration present in the native ink. Based on the measurements reported in the present study, the average concentrations of L-dopa and dopamine in squid ink are greater than those nec-

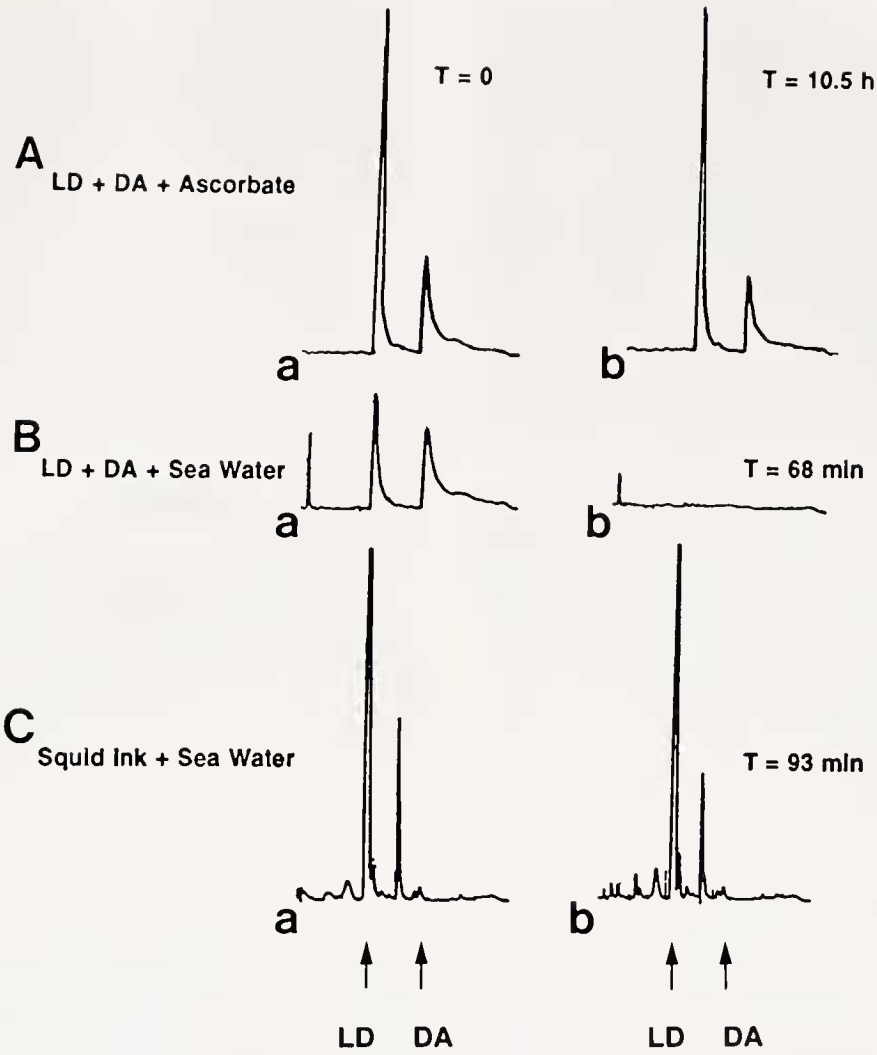

Figure 3. HPLC traces reveal that L-dopa (LD) and dopamine (DA) are stable in squid ink and in the presence of ascorbic acid, but oxidize rapidly in filtered seawater. (A) 100 μl of 50 μM L-dopa and dopamine in Na-acetate buffer containing 10 mM ascorbic acid was injected into the HPLC at time zero (a) and after 10.5 h at room temperature (b). (B) HPLC traces show rapid oxidation of 200 μl of 50 μM L-dopa and dopamine in seawater at time zero (a) and after 68 min at room temperature (b). The small sharp peak with an elution time of 0.6 min was assumed to be an oxidation product or contaminant and was not further characterized. (C) Squid ink diluted directly into seawater and injected into the HPLC at time zero (a) shows very little oxidation of L-dopa and dopamine after 93 min at room temperature, despite gassing for 25 min with O_2 (b).

essary to elicit behavioral (Gilly and Lucero, 1992) and physiological (Lucero *et al.*, 1992) responses and are therefore physiologically significant. Our work thus supports the idea that ink is an alarm substance and that products of melanin synthesis (L-dopa and dopamine) are present in the ink at levels that provide chemical signals to other squid. In addition, the levels of these biogenic amines vary considerably among individual ink sacs, and this variability is not due to differential oxidation of the ink during handling or to errors in measurement. Finally, we present preliminary evidence that a natural antioxidant that stabilizes these chemical messengers in seawater may be present in ink.

Both fluorescence and electrochemical detection methods were employed, after HPLC separation, to identify and quantify L-dopa and dopamine in squid ink. Both methods showed peaks in the ink that eluted at the same times and with the same characteristic shapes as L-dopa and dopamine standards. With fluorescence detection, the average concentrations for L-dopa were 1.15 ± 0.56 mM ($n = 10$), and for dopamine were 188 ± 67 μM ($n = 9$). L-dopa concentrations were also obtained by electro-

chemical detection that were as high as the lowest levels measured with the fluorescence (ink sac I), and the maximum dopamine concentration measured electrochemically was about 3 time lower than the lowest value found with fluorescence. But oxidation experiments with standards (Fig. 3A) showed that, over a period of 10.5 h in the presence of 10 m*M* ascorbic acid, dopamine oxidized more rapidly than L-dopa. Thus, the reduced concentrations of both compounds measured electrochemically probably reflect oxidation that occurs after the removal of antioxidants with the alumina silica column. Similarly, slow differential oxidation of L-dopa, dopamine, and DHBA may have occurred during handling, shipment, and storage of the ink that cannot be corrected for by the known amount of DHBA degradation.

The concentrations of L-dopa and dopamine measured with the fluorescence detector are higher than those needed to elicit chemosensitive behavioral responses from intact animals (Gilly and Lucero, 1992) or electrophysiological responses from sensory neurons isolated from the squid olfactory organ (Lucero *et al.*, 1992). Exact quantitative comparisons are difficult because the dilution associated with pressure ejection from a small pipette is uncertain. Published experiments have revealed that squid can detect a 1:20 dilution of the ink (Gilly and Lucero, 1992), suggesting that the detection of chemical stimuli by squid is quite sensitive.

Thus, even when ejected ink is diluted into seawater, these compounds will still be detectable by a squid, at least for a short time. The lifetime of this chemical alarm signal would presumably depend on the extent to which dopamine or L-dopa remain bound to the mucus-like components of the ink that are responsible for holding the ink mass together in compacted form. A valuable approach to the biological relevance of our findings will be to employ electrochemical probe analysis of natural ink plumes to measure the temporal and spatial characteristics of the dopaminergic signal in ejected ink (Moore and Atema, 1988; Moore *et al.*, 1989).

The concentrations of L-dopa and dopamine varied greatly among ink sacs, but the ink sac with the highest concentration of L-dopa also had the highest dopamine concentration, suggesting a metabolic linkage. This is not surprising given that dopamine is synthesized from L-dopa through the action of dopa decarboxylase (Stryer, 1981).

The variability in catecholamine concentration among ink sacs seems not to be due to rapid oxidation, because the data shown in Figure 3 indicate that L-dopa and dopamine oxidize much more slowly in diluted ink than do standards added to filtered seawater. One source of ink antioxidant may be the melanin pigments. Recent studies suggest that, although melanins are assumed to be remarkably stable, those synthesized from L-dopa are capable of self-oxidation under biologically relevant conditions (Aime and Fasano, 1990; Crescenzi *et al.*, 1993). This reductive capacity of melanin pigments has led to the suggestion that, in living systems, melanin plays a role in removing H_2O_2 (Aime and Fasano, 1990). Although the melanin pigments could be stabilizing L-dopa and dopamine in squid ink by preventing oxidation, another antioxidant may also exist, because the experiments in Figure 3 were run on ink from which the majority of melanin pigments had been physically removed by centrifugation and filtration. Perhaps, in addition to the melanin, an antioxidant such as ascorbic acid is released with the ink. In any case, the variability among ink sacs is not due to rapid oxidation and may depend on the length of time that the ink was stored in the ink sac. We did not attempt to record the inking history of the squid used in these experiments, but some squid inked upon capture and others did not.

In summary, these experiments show that squid ink contains high concentrations of L-dopa and dopamine. This study supports earlier findings that these biogenic amines are behaviorally (Gilly and Lucero, 1992) and physiologically (Lucero *et al.*, 1992) relevant olfactory stimuli. Further work will be necessary to identify the antioxidant in the ink, determine the source of variability between animals, and characterize the chemical message in a natural ink plume.

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Literature Cited

Aime, S. and M. Fasano. 1990. Solid-state ^{13}C NMR characterization of melanin free acids from biosynthetic and natural melanins. *Gazz. Chim. Ital.* **120:** 663–665.

Crescenzi, O., M. d'Ischia, A. Napolitano, and G. Prota. 1993. The alleged stability of dopa-melanins revisited. *Gazz. Chim. Ital.* **123:** 241–242.

Fox, D. L. 1976. *Animal Biochromes and Structural Colors.* University of California Press, Berkeley. Pp. 215–240.

Gilly, W. F., and M. T. Lucero. 1992. Behavioral responses to chemical stimulation of the olfactory organ in the squid *Loligo opalescens*. *J. Exp. Biol.* **162:** 209–229.

Gomez-Nino, A., B. Dinger, C. Gonzalez, and S. J. Fidone. 1990. Differential stimulus coupling to dopamine and norepi-

nephrine stores in rabbit carotid body type I cells. *Brain Res.* **525:** 160–164.

Jaeckle, W. B., and D. T. Manahan. 1989. Amino acid uptake and metabolism by larvae of the marine worm *Urechis caupo* (Echiura) a new species in anexic culture. *Biol. Bull.* **176:** 317–322.

Jimbow, K., Y. Miyake, K. Homma, K. Yasuda, Y. Izumi, A. Tsutsumi, and S. Ito. 1984. Characterization of melanogenesis and morphogenesis of melanosomes by physicochemical properties of melanin and melanosomes in malignant melanoma. *Cancer Res.* **44:** 1128–1134.

Lucero, M. T., F. T. Horrigan, and W. F. Gilly. 1992. Electrical responses to chemical stimulation of squid olfactory receptor cells. *J. Exp. Biol.* **162:** 231–249.

MacGinitie, G., and N. MacGinitie. 1968. *Natural History of Marine Animals,* 2nd ed. McGraw-Hill, New York. Pp. 395–397.

Manahan, D. T. 1989. Amino acid fluxes to and from seawater in axenic veliger larvae of a bivalve (*Crassostrea gigas*). *Mar. Ecol. Prog. Ser* **53(3):** 247–255.

Moore, P., and J. Atema. 1988. A model of a temporal filter in chemoreception to extract directional information from a turbulent odor plume. *Biol. Bull.* **174:** 355–363.

Moore, P. A., G. A. Gerhardt, and J. Atema. 1989. High resolution spatio-temporal analysis of aquatic chemical signals using microelectrochemical electrodes. *Chem. Senses* **14:** 829–840.

Prota, G., J. P. Ortonne, C. Voulot, C. Khatchadourian, G. Nardi, and A. Palumbo. 1981. Occurrence and properties of tyrosinase in the ejected ink of cephalopods. *Comp. Biochem. Physiol.* **68B:** 415–419.

Stryer, L. 1981. *Biochemistry,* 2nd ed. W. H. Freeman and Company, San Francisco. Pp. 895–896.

Waite, J. H. 1992. The DOPA ephemera: a recurrent motif in invertebrates. *Biol. Bull.* **183:** 178–184.